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An outbreak case of *Clostridium difficile*-associated diarrhea among elderly inpatients of an intensive care unit of a tertiary hospital in Rio de Janeiro, Brazil

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Abstract

The aim of this study was to investigate *Clostridium difficile*-associated diarrhea (CDAD) in an intensive care unit (ICU) of a tertiary hospital in Rio de Janeiro, Brazil, and to characterize epidemiologically *C. difficile* strains obtained from an outbreak of CDAD. Within almost a 4-year surveillance period, CDAD incidence was determined for the first time in Brazil, and a 3-fold increase was observed in the average rate of CDAD, featuring an outbreak. About 80% of the patients were over 65 years. The main antibiotic that could be probably associated to CDAD was piperacillin/tazobactam. Four toxigenic strains were isolated, 3 from stools and 1 from environmental samples. They were all resistant to clindamycin and fluoroquinolones. Fingerprinting analysis revealed their distribution between 2 different polymerase chain reaction ribotypes, with one of them being exclusively found in Brazil. It was possible to detect cross-infection and environmental contamination in the ICU. Our results highlight the importance of a continuous CDAD surveillance in the hospitals, especially when a risk group is exposed.

Keywords: *Clostridium difficile*; *Clostridium difficile*-associated diarrhea; Intensive care unit; Elderly inpatients

1. Introduction

Clostridium difficile infection (CDI) is a toxin-mediated intestinal disease ranging from asymptomatic colonization to mild diarrhea (*C. difficile*-associated diarrhea [CDAD]) and more severe clinical manifestations, such as pseudomembranous colitis, toxic megacolon, bowel perforation, sepsis, shock, and death (Rupnik et al., 2009).

The ability of *C. difficile*, an anaerobic Gram-positive bacillus, to form spores is considered a key feature in enabling it to persist in patients and in the physical environment for long periods, facilitating its dissemination (Poutanen and Simor, 2004). In fact, CDI represents the most common cause of nosocomial infectious diarrhea among hospitalized adults under antibiotic therapy (Leclair et al., 2010). The use of antibiotics disrupts the normal intestinal microbiota, leading to the colonization of the gut

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by *C. difficile*. Although exposure to almost all antimicrobials can be related to CDI, recent studies suggest that fluoroquinolones play an important role in this scenario, especially in the last 5 years, due to the emergence of the epidemic hypervirulent strain NAP1/027 associated to more life-threatening cases of CDAD (Hookman and Barkin, 2009; Riddle and Dubberke, 2009).

Besides antibiotic exposure, other major risk factors are associated to CDI. Old age (≥65 years), hospitalization, severe underlying diseases, and acid gastric suppression have been reported (Leclair et al., 2010; Rupnik et al., 2009). It is important to mention that many of these characteristics are often found in critically ill patients residing in intensive care units (ICU), making ICU stay another important risk factor for CDI development (Riddle and Dubberke, 2009).

Many researches have pointed out that CDAD incidence among the elderly (over 65 years) was up to 10 times higher than among younger adults, and that age-specific attributable mortality increases in this population, especially when they are at the ICU setting (Graf et al., 2009; Gravel et al., 2009; Marcon et al., 2006). This is generally thought to reflect a failure in the immune system as a result of comorbidities and normal age-related changes, known as senescence of the immune response. This phenomenon, associated to T-cell alterations, may explain the irresponsive status against *C. difficile* toxins (Leclair et al., 2010; Mitty, 2009).

Little is known about the incidence of *C. difficile* in Brazil, especially among the elderly in the ICU. The objectives of our study were to investigate retrospectively the CDAD cases in an ICU of a tertiary hospital located at Rio de Janeiro, Brazil, and to characterize phenotypically and genotypically *C. difficile* strains obtained from stools and environmental samples collected in an outbreak of CDAD in this setting.

2. Materials and methods

2.1. Setting

This study was carried out in a 30-bed medical surgical ICU of a tertiary hospital located at Rio de Janeiro city. The study protocol and data collection were approved by the institutional review board and were compliant with the hospital regulations.

2.2. CDAD incidence

Between January 2006 and July 2009, medical records of all ICU patients were reviewed retrospectively for clinical and demographic data. An incidence curve was obtained by using the Microsoft Office Excel software (Microsoft, Redmond, WA).

2.3. C. difficile isolates

Sample collection was carried out in the ICU from March to July 2008.

2.3.1. Stool samples

Samples were taken from inpatients at the description of the attending physician and tested for the presence of toxins A and B by Ridascreen *C. difficile* Toxin A/B (R-Biopharm, Darmstadt, Germany) immunoenzymatic assay (ELISA), according to the manufacturer's instructions. Stool samples were also cultured by using standard procedures (Jousimies-Somer et al., 2002). *C. difficile* isolates were identified based on their typical morphology on cycloserine cefoxitin fructose agar plates, based on their characteristic odor, and by Gram staining. Conventional biochemical tests (Jousimies-Somer et al., 2002) and the polymerase chain reaction (PCR) targeting *tpi* gene (Lemee et al., 2004) were used to confirm the identification.

2.3.2. Environmental samples

Surfaces of the ICU were screened (lavatory sinks, medical instruments, hospital monitors, floors, windows and doorknobs, bedpans, washstands) by using sterile premoistened cotton swabs, which were transported to the laboratory and immediately cultured at cycloserine cefoxitine fructose (CCF) broth. The procedures used to *C. difficile* isolation and identification were the same as described above.

2.4. Toxins detection

PCRs to detect the toxigenic profile of isolates, concerning the presence of *tcd*A (Lemee et al., 2004), *tcd*B (Balassiano et al., 2009), *cdt*A, and *cdt*B (Barbut et al., 2007) genes, were done as previously described. Amplification and sequencing of *tcd*C (Spigaglia and Mastrantonio, 2004) were also performed to investigate the presence of possible deletions at this gene. Sequences were compared using the BLAST server of the National Center for Biotechnology Information (NCBI).

2.5. Molecular fingerprinting analysis

2.5.1. Pulsed field gel electrophoresis

The genetic fingerprint of the *C. difficile* isolates was determined by pulsed field gel electrophoresis, according to Balassiano et al. (2009). Briefly, agarose plugs were made by mixing 3×10^9 CFU/mL of freshly grown bacterial cell suspension (37 °C for 7 h in petone-yeast-glucose broth) resuspended in Gram-positive lysis buffer (6 mmol/L Tris–HCl, pH 8.0; 1 mol/L NaCl; 100 mmol/L EDTA, pH 8.0; 0.5% Brij 58; 0.2% sodium deoxycholate; 0.5% sodium lauryl sarcosine) with 2% low melting point agarose (Sigma, St. Louis, MO). Plugs were incubated overnight at 37 °C in Gram-positive lysis buffer containing 5 mg/mL lysozyme (Sigma) and 100 μg/mL RNase A (Sigma), and subsequently incubated overnight at 51 °C with a fresh solution containing 0.5 mol/L EDTA ,pH 8.0 (Sigma), 1% sodium dodecyl sulfate (Sigma), and 100 μg/mL proteinase K (Sigma). Washed plugs were digested with 15 U of *Sma*I (New England Biolabs, Ipswich, MA) for 4 h at 22 °C, and DNA fragments were separated in 1% Ultra Pure agarose (Invitrogen, São Paulo, Brazil) in

Table 1
CDAD incidence during the 43-month surveillance period in an ICU of a tertiary hospital in Rio de Janeiro

Month[a]	Patients/day (hospital)	No. of cases (ICU)	CDAD incidence (new cases/1000 patient day)
April 2006	458	1	2.2
April 2007	430	1	2.3
November 2007	598	1	1.7
December 2007	653	5	7.7
January 2008	625	4	6.4
February 2008	600	3	5.0
March 2008	578	2	3.5
April 2008	752	1	1.3
May 2008	700	1	1.4
June 2008	711	7	9.8
July 2008	603	6	10.0
August 2008	597	3	5.0
September 2008	543	1	1.8
December 2008	546	1	1.8
January 2009	553	1	1.8
March 2009	671	1	1.5
April 2009	570	1	1.8
May 2009	716	1	1.4
June 2009	638	1	1.6
July 2009	638	1	1.6
Total	23,468[b]	43	1.8[c]

[a] Months where no CDAD cases were reported are not listed in the table.
[b] Total number of patients/day included months that were not listed in the table.
[c] Average rate of CDAD during the overall study period.

0.5× Tris–boric acid EDTA buffer containing 200 μmol/L of thiourea. Gel images were analyzed with Gel Compar II Software (version 4.0; Applied Maths, Kortrijk, Belgium). The NAP1/027 *C. difficile* strain, kindly provided by Dr Angela Thompson, from Centers for Disease Control, Atlanta, GA, was used for genetic profile comparison.

2.5.2. PCR ribotyping

C. difficile isolates were analyzed at the Anaerobe Reference Laboratory by PCR ribotyping, according to Stubbs et al. (1999). Gel images were analyzed with Gel Compar II Software (version 4.0; Applied Maths, Kortrijk, Belgium).

2.6. Antibiotic susceptibility testing

MICs were determined by using E-test strips (AB Biodisk, Solna, Sweden). Briefly, after an overnight grown in prereduced *Brucella* broth, cultures were diluted to a 1-McFarland standard and immediately swabbed on pre-reduced *Brucella* blood agar plates supplemented with hemin and vitamin K. E-test strips were applied to the agar surface according to the manufacturer's instructions, and plates were incubated in anaerobic environment at 37 °C for 48 h (Barbut et al., 2007). Quality control strain (*Bacteroides fragilis* ATCC 25285) was used. The breakpoints for clindamycin (≥8 mg/L) and metronidazole (≥8 mg/L) were considered according to Clinical and Laboratory Standards Institute (2007) guidelines. For antimicrobial agents to which no standard breakpoints to *C. difficile* have been defined, breakpoints were considered as follows: vancomycin ≥8 mg/L, moxifloxacin ≥4 mg/L, levofloxacin ≥4 mg/L, and ciprofloxacin ≥8 mg/L (Huang et al., 2009a; Mutlu et al., 2007).

2.7. Detection of resistance genes and mutations

A quinolone resistance-determining region (QRDR) of *gyr*A and *gyr*B (Dridi et al., 2002) genes was amplified, and both strands of the resulting amplicons DNAs were purified (Wizard SV Gel and PCR Clean-Up System, Promega, Madison, WI) and sequenced. ClustalW amino acid sequence alignments were produced for comparison with *C. difficile* strain 630, whose sequences are available at NCBI Web site.

3. Results

During the 43-month surveillance period in the ICU, 218 patients presenting diarrhea (defined as a stool sample that took the shape of the sample container) had their stools tested for the presence of toxins. A total of 43 (19.7%) CDAD cases were confirmed, all representing nosocomial (ICU) infection. The average rate of CDAD was calculated as 1.8 per 1000 patient days, with the highest incidence between December 2007 and August 2008 (average, 5.5 per 1000

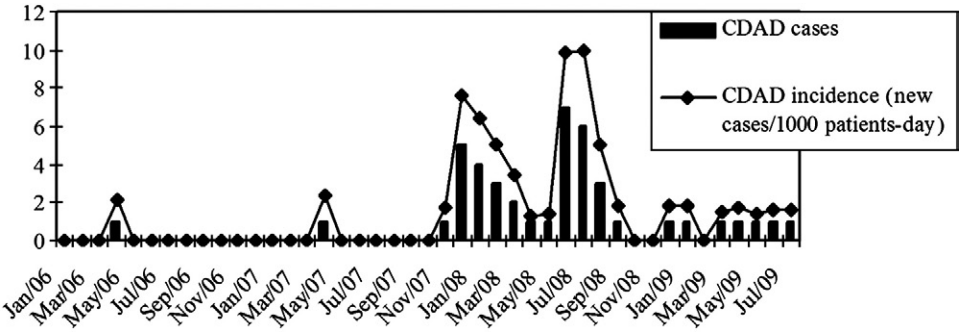

Fig. 1. CDAD incidence during the 43-month surveillance period in an ICU of a tertiary hospital in Rio de Janeiro.

Table 2
Characterization of the *C. difficile* strains isolated during an outbreak of CDAD in an ICU of a tertiary hospital in Rio de Janeiro, Brazil

Isolate identification	Toxigenic profile	PCR ribotype	MIC (mg/L)						Period of isolation
			MTZ	VAN	CLI	MX	LX	CIP	
SJ1 (female/68 years/SH, DM, hemorrhagic EVA)	*tcd*A+/*tcd*B+, *tcd*C no deletions, CDT−	135	0.19	1.0	6.0	0.5	>32.0	>32.0	March 2008
SJ4 (Male/88 years/EVA, ARDS, CAD)	*tcd*A+/*tcd*B+, *tcd*C no deletions, CDT−	038	0.25	0.75	>256.0	1.5	>32.0	>32.0	June 2008
SJ8 (Male/83 years/ARDS, neoplasia)	*tcd*A+/*tcd*B+, *tcd*C no deletions, CDT−	135	0.38	1.0	6.0	0.75	>32.0	>32.0	June 2008
Amb3 (ICU lavatory sink)	*tcd*A+/*tcd*B+, *tcd*C no deletions, CDT−	038	0.25	0.75	>256.0	1.0	>32.0	>32.0	June 2008

Toxigenic profile determined by PCR: *tcd*A (TcdA enterotoxin), *tcd*B (TcdB enterotoxin), *tcd*C (deletions at the negative regulator gene that controls TcdA and TcdB production), CDT (binary toxin, related to *cdt*A and *cdt*B genes).
SH = systemic hypertension; DM = diabetes mellitus; EVA = hemorrhagic encephalic vascular accident; ARDS = acute respiratory distress syndrome; CAD = coronary artery disease; MTZ = metronidazole (breakpoint ≥8 mg/L); VAN = vancomycin (breakpoint ≥8 mg/L); CLI = clindamycin (breakpoint ≥8 mg/L); MX = moxifloxacin (breakpoint ≥4 mg/L); LX = levofloxacin (breakpoint ≥4 mg/L); CIP = ciprofloxacin (breakpoint ≥8 mg/L).

patient days), which featured an outbreak of *C. difficile* in this period (Table 1 and Fig. 1). During the outbreak, stools of 101 patients were tested, and toxins were detected in 32 (31.5%) of them. No severe cases of CDAD occurred, and no attributable mortality due to CDAD was observed in the outbreak. In general, the patients with CDAD during the outbreak were elderly, with 80% grater than 65 years, with average age of 77.8 years. Concerning some criteria to assess disease severity, the average Acute Physiology and Chronic Health Disease Classification System II (APACHE II) score was 20.7 point (standard deviation, 5.3), and the average Sepsis-related Organ Failure Assessment (SOFA) score was 4.2 point (standard deviation, 3.1).

Records of the medical analysis of the ICU patients enrolled in the outbreak revealed that some antimicrobial classes were more frequently used before the onset of diarrhea. Piperacillin/tazobactam was used in 81% (26/32) of the patients, teicoplanin in 65.6% (21/32), carbapenems in 59.3% (19/32), quinolones in 56.3% (18/32), and cephalosporins in 50% (16/32) of them. The same antimicrobials were also more routinely applied as therapeutic agents during all the period of the study.

In order to characterize epidemiologically the CDAD outbreak in the ICU setting of the hospital, stool and environmental samples were collected, aiming the isolation of *C. difficile*. Not all ELISA-positive patients had their stool samples submitted for culture, and not all submitted samples (stool and environmental) yielded a toxigenic *C. difficile* strain when cultured. The bacterium could be recovered from 3 of the 13 ELISA-positive stool samples analyzed and from 1 of the 40 environmental samples.

Amplification reactions revealed that the 4 isolates presented a toxigenic profile concerning the presence of toxins A and B genes (*tcd*A and *tcd*B). Neither genes of the binary toxin (*cdt*A and *cdt*B) were detected nor any significant deletions in *tcd*C gene were observed.

Molecular fingerprinting analysis (Table 2 and Fig. 2) revealed that the 4 isolates were distributed among the following different PCR ribotypes and pulsotypes: 135/pulsotype B (strains SJ1 and SJ8) and 038/pulsotype A (strains SJ4 and Amb3), respectively. None of them belonged to the hypervirulent clone NAP1/027.

Concerning the antimicrobial resistance profile, the strains were sensitive to metronidazole, vancomycin, and moxifloxacin and resistant to clindamycin, ciprofloxacin, and levofloxacin (Table 2). To investigate a possible relation between the resistance to the fluoroquinolones ciprofloxacin and levofloxacin and mutations at *gyr*A and *gyr*B genes, we amplified and sequenced specific regions of these genes, but amino acid analysis did not revealed any significant amino acid substitutions that could explain the resistance (data not shown).

4. Discussion

Nosocomial diarrhea is a common problem in critical ill patients regardless of the disease process that necessitated

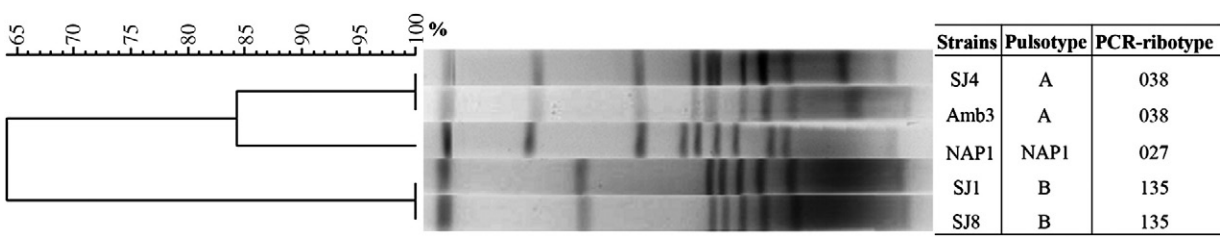

Fig. 2. Pulsotypes and PCR ribotypes of *C. difficile* strains isolated during an outbreak of CDAD in an ICU of a tertiary hospital and comparison with NAP1/027 strain.

admission to the ICU, and this scenario can get worst if we consider that in this setting, patients are generally exposed to many factors that lead to the reduction of their immune defenses, aggravating the diarrhea (Marcon et al., 2006; Riddle and Dubberke, 2009). Overall, up to 40% of the patients develop diarrhea after ICU admission, which can be associated to different aspects, such as enteric feeding, hypoalbuminemia, intestinal ischemia, medications, or infections. In the last case, the most important infectious cause is CDAD (Riddle and Dubberke, 2009).

CDAD becomes a more alarming situation when it affects elderly inpatients (age ≥65 years). Zilberberg et al. (2008) noticed that between 2000 and 2005, the prevalence of CDAD among this specific age group more than doubled. Another study showed a 10-fold increase in the incidence of CDAD among older inpatients between 1991 and 2003 (Pépin et al., 2004). Besides, elderly patients with CDAD were considered responsible for the increased hospital mortality rates, presenting a higher 30-day mortality than younger patients in the ICU setting (Zilberberg et al., 2009).

In the present work, we analyzed the incidence of CDAD among elderly inpatients admitted at the ICU of a tertiary hospital in the Rio de Janeiro city. In Brazil, information regarding the incidence of CDAD and the spread of *C. difficile* inside hospital units is still limited, making our study brand new in this field. Our data corroborate with other authors that demonstrate the prevalence of older inpatients presenting CDAD in the ICU setting (Marcon et al., 2006; Zilberberg et al., 2009).

We found an average rate of CDAD as 1.8 per 1000 patient days during the 43-month period. Goldstein et al. (2009) described a rate of nosocomially acquired CDAD in a long-term acute care facility in Los Angeles, as 3.12 per 1000 patient days during their 1-month study. Gravel et al. (2009) showed 4.6 cases per 1000 patient admission during the 6-month surveillance period in 13 hospitals in Canada. Unfortunately, we do not have available data to compare the CDAD incidence in Brazilian hospitals. However, it is important to highlight that previous studies have suggested that antibiotic usage, the physical layout of the institution, and both the infection prevention and infection control practices have played an important role in the overall incidence of CDAD inside a particular hospital unit (Gravel et al., 2009).

Concerning the antimicrobial therapy prior to the onset of diarrhea, all the antibiotics used by the ICU patients were those previously considered at high risk for CDAD development (Hookman and Barkin, 2009; McFarland, 2008). Despite that some authors suggest the importance of fluoroquinolones as one of the most important antimicrobial classes inducing CDAD in the last decades (Ackermann et al., 2003; Riddle and Dubberke, 2009), we believe that in our case, this was not the most important inducer, but piperacillin in association with tazobactam. We also found out an important association between carbapenems (especially meropenem) and diarrhea, corroborating with recent work from Metzger et al. (2010). It is important to highlight that the implication of almost every antibiotic in CDAD development reflects widespread use and also the difficulty in attributing the relative risk of this infection to specific antibiotics (Freeman et al., 2010).

Between December 2007 and August 2008, we observed a 3-fold increase in the average rate of CDAD (from 1.8 to 5.5 per 1000 patient days). In order to control this outbreak of CDAD in the ICU, an infection control program to reduce the spread of CDAD was adopted: intensive education of staff; use of chlorhexidine-based hand sanitizers instead of water and soap and alcohol gel; contact precautions until patient discharge instead of contact precaution after 48 h of the absence of symptoms; use of potassium monopersulfate (1% active chlorine) to surface disinfection instead of hypochlorite; rational use of antimicrobials; immediate alert of the laboratory; substitution of the toxin A detection kit to another that detects both toxins A and B; weekly screening of patients presenting unmolded feces for toxins detection; and sterilization or discard of items used by toxin positive patients.

In order to better understand the epidemiologic characteristics of the CDAD outbreak, stool and environmental samples were collected during this period, aiming the isolation of *C. difficile*. Even though our work presented a small sampling, we could isolate 4 toxigenic *C. difficile* strains, 1 from the lavatory sink of the ICU and 3 from stools of different patients. It is important to notice that these patients presented some aspects that are often associated with the emergence of CDAD, such as advanced age, antibiotic therapy, immune suppression condition due to underlying diseases and/or the therapy associated to these diseases, and admission at ICU (Leclair et al., 2010).

Although the persistence of *C. difficile* in the environment is well documented, with reports of isolation from environmental swabs taken from a patient's room months after discharge (Kim et al., 1981), the microorganism is typically thought to spread through person-to-person transmission, with health care workers often being responsible for spreading the organism on their hands or medical equipment (Riddle and Dubberke, 2009). This fact could explain our low rate (1/40) of environmental recovery of *C. difficile* in the collection realized in the ICU during the outbreak, since the health care staff was not included in our research.

Despite the small number of isolates obtained, we could detect cross-infection among 2 patients, where PCR ribotype 135 was isolated (SJ1 and SJ8 strains). This PCR ribotype had already been detected by our group in a previous work realized with pediatric inpatients (Alcides et al., 2007), and since it was never isolated in any other country, it is considered as an exclusively Brazilian ribotype. Besides cross-infection, we could also detect environmental contamination, since isolates belonging to PCR ribotype 038 were detected in both environmental (Amb3 strain) and stool (SJ4 strain) samples. These 2 distinct situations could be a reflection of failures in the protocols of environment disinfection and hygiene of the

hands of health care professionals at the time of the outbreak, which was controlled only when the infection control program was adopted, as previously described. It is important to note that cross-infection and environmental contaminations are 2 characteristics extremely crucial to the emergence of outbreaks associated with *C. difficile* (Hookman and Barkin, 2009).

Concerning the resistance profile, the isolates were all sensitive to metronidazole and vancomycin, as already expected. Some of the reasons are the high activity demonstrated against *C. difficile*, the rare reports of resistance, and the fact that these are the antimicrobials of choice for CDAD treatment (Huang et al., 2009b). The strains were also sensitive to moxifloxacin, which can be explained by the fact that this drug is considered 3- to 4-fold more active against *C. difficile* than other fluoroquinolones (Mutlu et al., 2007). On the other hand, the isolates exhibited resistance to clindamycin, ciprofloxacin, and levofloxacin, as already described by other authors (Barbut et al., 2007; Bourgault et al., 2006; Huang et al., 2009a).

Considering the emergent importance of fluoroquinolones in inducing CDAD cases (Hookman and Barkin, 2009), we decided to investigate the molecular mechanisms associated to resistance of the isolates to ciprofloxacin and levofloxacin. To achieve this goal, we analyzed specific regions of the genes that encode the 2 subunits of DNA gyrase enzyme (*gyr*A and *gyr*B genes), called QRDR. This specific region was chosen because alterations in this enzyme, especially due to amino acid substitutions at QRDR, can lead to a decrease in the affinity for the drug, which is considered to be the main mechanism of resistance to fluoroquinolones in different bacterial species, including *C. difficile* (Dridi et al., 2002; Huang et al., 2009b). Unfortunately, we did not identify any significant amino acid substitutions that could explain the resistance. A reasonable explanation is that the amino acid substitutions at QRDR are intrinsically associated to resistance to moxifloxacin (Ackermann et al., 2003), which was not observed in our isolates, despite the resistance to the other fluoroquinolones tested. Future studies will be conducted in order to investigate if other molecular mechanisms could be involved in the resistance to these drugs, such as expression of efflux pump systems, reduced permeability of the membrane, or amino acid changes outside the QRDR (Spigaglia et al., 2008).

In conclusion, we demonstrated for the first time in Brazil the incidence data about CDAD in the ICU setting of a tertiary hospital, with a prevalence of elderly inpatients. We also detected an outbreak during the study period, which was controlled when a combination of control measures were adopted. This CDAD outbreak could be epidemiologically characterized by the isolation and identification of phenotypic and genotypic aspects of the strains obtained. We showed cross-infection and environmental contamination, which can be considered an alarming situation that can lead to the emergence of an outbreak. Our results highlight the importance of a careful and continuous CDAD surveillance inside the hospitals, especially when a risk group is exposed.

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